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DEVELOPMENT OF NOVEL SYNTHETIC LUNG SURFACTANTS FOR TREATMENT OF RESPIRATORY DISTRESS SYNDROME

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DEVELOPMENT OF NOVEL SYNTHETIC LUNG SURFACTANTS FOR TREATMENT OF RESPIRATORY DISTRESS SYNDROME

Thesis for doctoral degree (Ph.D.)

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*To my truly missed father
and my family*

ABSTRACT

Pulmonary surfactant is a complex mixture of lipids and specific proteins found in the alveoli and its main role is to reduce the surface tension of the alveolar air/water interface, thereby preventing lung collapse. Lack of pulmonary surfactant in premature babies results in lethal respiratory distress syndrome (RDS), which is today treated with surfactant replacement therapy, i.e. delivery of pulmonary surfactant extracted from animal lungs into the airways. Despite the effectiveness of surfactant replacement therapy, it has several limitations: pronounced batch to batch variability, risk of disease transmission, and expensive source material and production procedures. Moreover, RDS secondary to surfactant inhibition in adults (ARDS) has turned out to be difficult to treat with existing natural derived surfactant preparations, probably for a combination of reasons, such as limited supply and sensitivity to inhibition. The development of synthetic pulmonary surfactants is a tempting solution that could overcome these limitations, but in spite of more than 30 years of development work, there still no synthetic surfactant in clinical use.

The two surfactant proteins B (SP-B) and C (SP-C) that are present in natural derived surfactants are difficult to produce or replicate and the lipid composition of natural surfactants is very complex. As a result, the development of simple lipid compositions and surfactant protein analogues that can reproduce the activity of animal derived surfactants is not trivial. In this thesis, we designed surfactant protein analogues and tested their activities in animal models of neonatal RDS and ARDS. We investigated the importance of intramolecular disulfide bonds in an SP-B analogue, and we improved the recombinant production of the SP-C analogue SP-C33Leu. Artificial surfactant based on recombinant SP-C33Leu improved lung mechanics in an RDS model and enhanced lung function and reduced inflammation in an ARDS model.

Finally, we investigated the novel concept of creating surfactant protein analogues that combine both SP-B and SP-C activities in one polypeptide. We designed and recombinantly produced polypeptides that fuse an SP-B analogue and an SP-C analogue - Combo peptides - and tested mixtures of these analogues with two phospholipid species in an RDS model ventilated without stabilizing end expiration pressures in the lung. Mixtures containing low amounts of Combo peptides improve lung function in a similar manner as a clinically used natural derived surfactant. The Combo peptide approach can deliver simple synthetic surfactants that match currently used natural preparations and can be produced efficiently.

LIST OF SCIENTIFIC PAPERS

- I. **Basabe-Burgos, O.**, Johansson, J. and Curstedt, T. *Disulphide bridges in surfactant protein B analogues affect their activity in synthetic surfactant preparations*. Neonatology 115, 134-141 (2019).
- II. **Basabe-Burgos, O.**, Zebialowicz Ahlstrom, J., Mikolka, P., Landreh, M., Johansson, J., Curstedt, T. and Rising, A. *Efficient delipidation of a recombinant lung surfactant lipopeptide analogue by liquid-gel chromatography*. PLoS One 14, e0226072 (2019).
- III. Zebialowicz Ahlstrom, J., Massaro, F., Mikolka, P., Feinstein, R., Perchiazzi, G., **Basabe-Burgos, O.**, Curstedt, T., Larsson, A., Johansson, J. and Rising, A. *Synthetic surfactant with a recombinant surfactant protein C analogue improves lung function and attenuates inflammation in a model of acute respiratory distress syndrome in adult rabbits*. Respir Res 20, 245 (2019).
- IV. **Basabe-Burgos, O.**, Landreh, M., Rising, A., Curstedt, T. and Johansson, J. *Treatment of respiratory distress syndrome with synthetic surfactants containing phospholipids and single polypeptide analogues that combine features of SP-B and SP-C*. Manuscript.

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LIST OF ABBREVIATIONS

ALI	Acute lung injury
ARDS	Acute respiratory distress syndrome
A β	Amyloid- β
BAL	Bronchoalveolar lavage
CD	Circular dichroism
CRD	Carbohydrate recognition domains
DPPC	Dipalmitoylphosphatidylcholine (=1,2-dipalmitoyl- <u>sn</u> -glycero-3-phosphocholine)
ER	Endoplasmic reticulum
FTIR	Fourier transform infrared
IAPP	Islet amyloid polypeptide
ILD	Interstitial lung disease
LB	Lamellar bodies
MVB	Multivesicular bodies
NT	N-terminal domain
PC	Phosphatidylcholine
PEEP	Positive end expiratory pressure
PG	Phosphatidylglycerol
POPG	Palmitoyl-oleyl-phosphatidylglycerol(=1-palmitoyl-2-oleoyl- <u>sn</u> -glycero-3-phosphoglycerol)
RDS	Respiratory distress syndrome
SAPLIP	Saposin-like protein
SDS	Sodium dodecyl sulfate
SP	Surfactant protein
TM	Tubular myelin
VILI	Ventilator induced lung injury

Three and one letter abbreviations of the 20 genetically encoded amino acid residues

<u>Amino acid</u>	<u>Three letter code</u>	<u>One letter code</u>
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

1 INTRODUCTION

1.1 Background

The breathing cycle and the tree-like structure of the lungs lay the foundation for gas exchange. The airways are branched 23 times (1) from the trachea to the alveoli, producing a large area of 50-100 m² exposed to air in an adult lung, and normal breathing refreshes part of the air in the airways and alveoli every 3-5 seconds. There is an air/water interface lining the alveoli and since the forces between neighboring water molecules are stronger than the forces between the water and the gas phase, a surface tension is created that promotes lung collapse (von Neergaard, 1929). However, under physiological conditions surface active agents are able to reduce the alveolar surface tension to near zero values, which markedly facilitates lung expansion and reduces the efforts of respiration (Gruenwald, 1947). In other words, creation of a low alveolar surface tension is a prerequisite for normal lung function (2).

In the 1950s two researchers, Pattle and Clements, independently identified the surface-active agent referred to as lung or pulmonary surfactant, which reduces the surface tension of the air/water interface in the mature alveoli (3, 4). Shortly thereafter, the lack or insufficient amounts of lung surfactant was associated to what was then called hyaline membrane disease by Avery and Mead (5). The hyaline membrane disease is nowadays known as respiratory distress syndrome (RDS) and is primarily associated with premature birth, usually before 30 gestational weeks, when the babies have immature lungs that are not able to produce sufficient amounts of lung surfactant. Even though the cause of RDS was established at this time, the development and clinical implementation of a treatment of this lethal condition took over two decades.

The first attempts to treat RDS in clinical trials employed instillation of dipalmitoylphosphatidylcholine (DPPC) based lipid mixtures, and were unsuccessful (6). Even though DPPC is the major component of pulmonary surfactant and is able to reduce surface tension to low values *in vitro*, it needs to be combined with proteins and other phospholipids to show rapid enough spreading properties *in vivo* (7). In 1972 Enhorning and Robertson showed that tracheal instillation of natural-derived surfactant, extracted from adult animal lungs was able to increase lung expansion in premature rabbits (8).

The first successful clinical study with human babies with RDS using bovine natural-derived surfactant instillation was performed just a few years after the first demonstration of efficacy in animals, and was published in 1980 by Fujiwara and coworkers (9). At about the same time,

at Karolinska Institutet Robertson and Curstedt developed a surfactant preparation derived from porcine lung, which is nowadays known under the name Curosurf[®] (10). Curosurf[®] was tested successfully in randomized clinical trials in the 1980s, which proved the efficacy and optimal dosage against neonatal RDS (11, 12). Several other natural derived pulmonary surfactant preparations, for example Survanta[®], Infasurf[®] and Alveofact[®], prepared from different animal sources, purified in slightly different ways, and with somewhat different compositions in terms of phospholipid profiles and protein contents, were also developed and tested successfully in clinical trials, see (13-22). Natural derived surfactant preparations are still in clinical use, having saved the lives of thousands of babies per year worldwide since their introduction. There are still, however, several reasons to develop synthetic lung surfactants for treatment of RDS, which will be described further below.

1.2 Lung surfactant biosynthesis

In the alveolar region we can observe three major types of cells: alveolar macrophages and type I and type II alveolar epithelial cells. The macrophages take part in lung host defense and can phagocytize inhaled particles, invading microorganisms, and also remnants of alveolar pulmonary surfactant (23). The alveolar type I epithelial cells form the main part of the alveolar wall. They are elongated and thin cells that contribute most of the alveolar surface area (90-95%) and mediate the diffusion of gas between inhaled air and the pulmonary capillaries (24). The alveolar type II cells make up the remaining 5-10% of the alveolar wall, are globular in shape and, importantly, produce and secrete lung surfactant components (25).

In the alveolar type II cells, pulmonary surfactant lipids and proteins are synthesized in the endoplasmic reticulum (ER), transferred to the Golgi apparatus for modifications like attachment of glycosyl moieties, and then transported to multivesicular bodies (MVB), which eventually mature into the intracellular storage form of surfactant - lamellar bodies (LB) (26) (Figure 1). As the name implies, the MVB are vesicles where the outer membrane invaginates and forms smaller vesicles within, the MVB is thus a vesicle with multiple vesicles inside. During the formation of the MVB, the complex and unique lipid and protein composition of pulmonary surfactant is established. The LB are onion-like structures in the sense that they contain multiple tightly packed phospholipid bilayers. The LB are transported out of the alveolar type II cells and into the alveoli by exocytosis, where the densely packed bilayers unpack, and turn into a more loosely organized network called tubular myelin (TM). Eventually the pulmonary surfactant phospholipids will form a monolayer at the air/water interface, in

which the hydrophobic phospholipid fatty acyl tails face air and the hydrophilic phospholipid head groups face the water layer (27). (Figure 1)

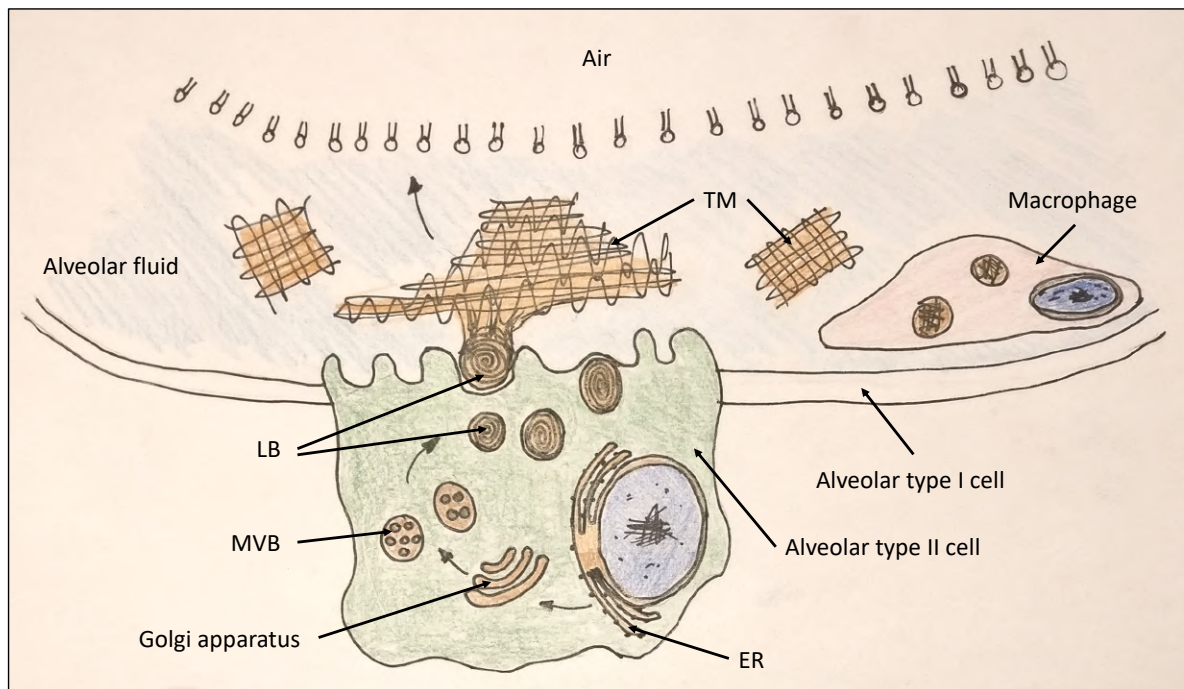


Figure 1. Alveolar components and pulmonary surfactant production. The alveolus is defined by and contains three different types of cells: alveolar macrophages that phagocytize particles including residual surfactant and microorganisms, alveolar type I cells that mediate oxygen and carbon dioxide diffusion and form the major part of the alveolar wall, and alveolar type II cells that produce and secrete pulmonary surfactant. Pulmonary surfactant is produced in the endoplasmic reticulum (ER) and travel through the Golgi apparatus to form multivesicular bodies (MVB) and eventually lamellar bodies (LB). The LB are transported out of the cell by exocytosis, where after the tubular myelin (TM) is formed and eventually phospholipids, and probably proteins, form a monolayer at the air/water interface.

1.3 Lung surfactant composition

The composition of the surface-active pulmonary surfactant was initially believed to be only lipids until, in the 1970s, small amounts of proteins were observed in natural surfactant (7, 28). Years later the hydrophobic surfactant protein (SP)-B and SP-C were shown to be part of the organic solvent extracts of lung tissue or bronchoalveolar lavage that were used to produce natural derived surfactant preparations (29). Nowadays is known that pulmonary surfactant

obtained by bronchoalveolar lavage of mammals consists of about 80% (w/w) phospholipids, 10% neutral lipids (mostly cholesterol) and 10% proteins (30) (Figure 2).

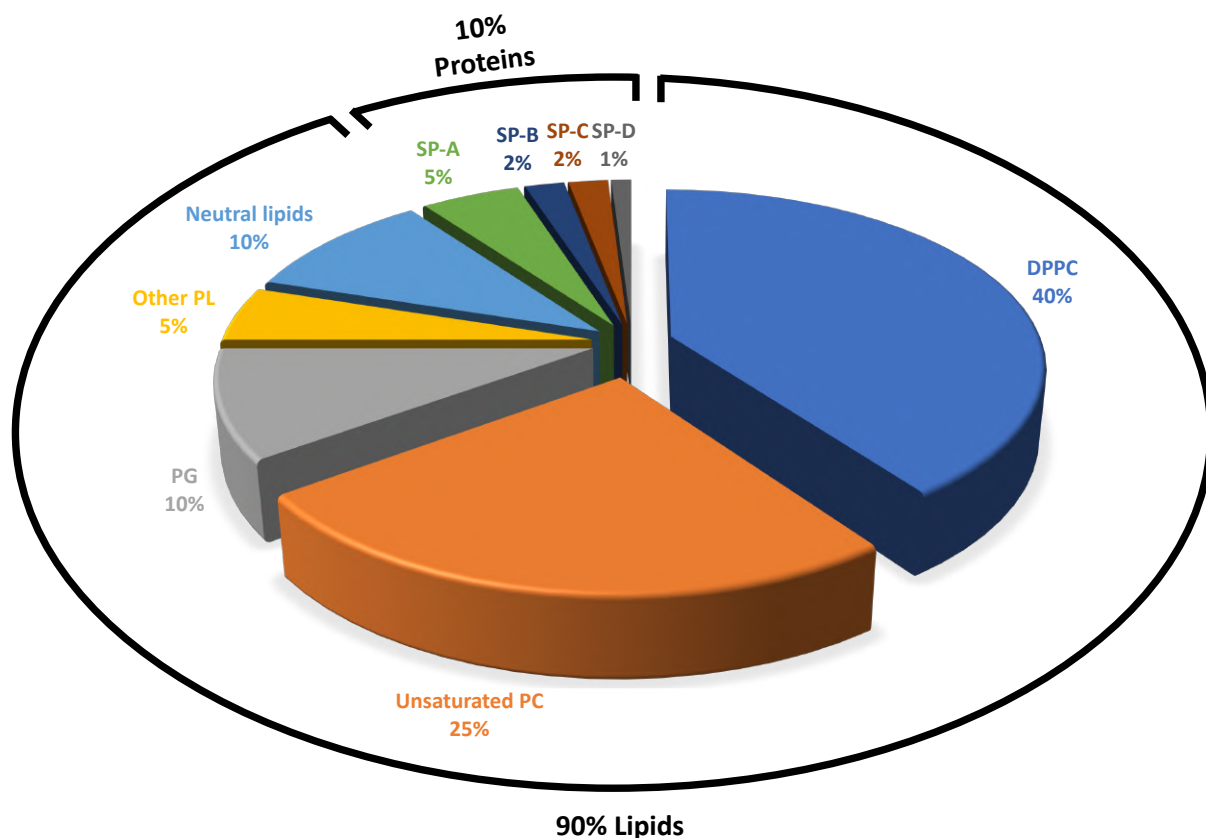
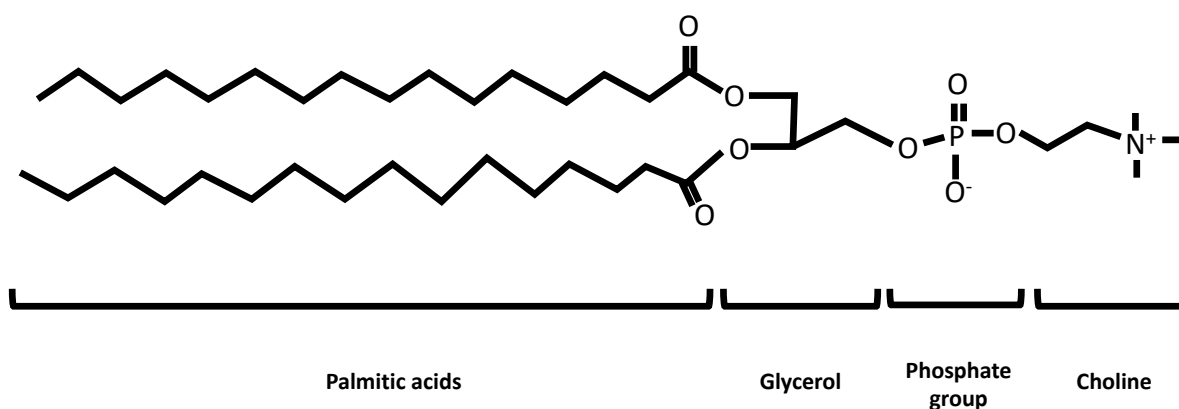


Figure 2. Composition of pulmonary surfactant. DPPC, dipalmitoylphosphatidylcholine; PC: phosphatidylcholine; PG: phosphatidylglycerol; PL, phospholipids; SP-A, -B, -C, -D, surfactant protein -A, -B, -C, -D. The percentages are by mass.

Phospholipids are amphiphilic molecules, i.e. they have one polar part and one non-polar part, that consist of a glycerol skeleton linked to one hydrophilic phosphate group, which in turn is esterified with an alcohol group, and to two hydrophobic fatty acyl chains. The fatty acyl chains are linked via hydroxyl esters to glycerol and contain either only single bonds (saturated fatty acyl chains) or contain also carbon-carbon double bonds (unsaturated fatty acyl chains). The different combinations that can be formed by esterifying the phosphate group at position 3 of the glycerol moiety with one of five alcohols (glycerol, choline, ethanolamine, serine or inositol) combined with the attachment of different fatty acyl tails to positions 1 and 2 of the glycerol moiety result in that more than 50 different phospholipid species are found in lung surfactant of mammals (31). However, the main single component of lung surfactant (about 40% by mass) is DPPC, which is present only in minimal amounts in other phospholipid

membranes, and therefore the high percentage of DPPC is in principle unique to lung surfactant. DPPC contains two saturated fatty acyl chains (palmitoyl groups, with 16 carbon atoms) (Scheme 1) and is mainly responsible for reducing the surface tension at the alveolar air/water interphase. Pulmonary surfactant also contains about 25% by mass of unsaturated phosphatidylcholine (PC) species, 10% of phosphatidylglycerol (PG) species and about 5% of other phospholipid species (phosphatidylinositols, phosphatidylserines and phosphatidylethanolamines) (32). The high percentage of PG species is, like the high DPPC content, an apparently unique feature of lung surfactant compared to other phospholipid membranes, and strongly suggest that these phospholipids have specific and important functions that are necessary for lung surfactant activity.



Scheme 1. Molecular structure of the saturated phospholipid DPPC. DPPC is the most abundant molecule in pulmonary surfactant and has a melting temperature of 41°C, which means that DPPC bilayer membranes are too rigid for efficient spreading in the alveoli at physiological body temperatures of mammals, but DPPC can efficiently reduce surface tension under *in vitro* conditions when the rate of spreading is not limiting.

The protein content in pulmonary surfactant is divided between four specific proteins: about 6% by mass of the pulmonary surfactant dry weight is made up of the multimeric and hydrophilic proteins SP-A and SP-D and about 4% of lung surfactant mass is made up of the comparatively small and hydrophobic proteins SP-B and SP-C (30, 33-36) (Table 1).

Table 1. Characteristics of lung surfactant proteins

	Solubility properties	Monomer mass	Protein subunits	Family	Main role	Dry weight in native surfactant
SP-A	Hydrophilic	~35 kDa	18	Collectin	innate pulmonary immune response	~5 %
SP-B	Hydrophobic	8.7 kDa	2	Saposin-like	reduce surface tension in the alveoli	~2 %
SP-C	Hydrophobic	4.2 kDa	1	-	reduce surface tension in the alveoli	~2 %
SP-D	Hydrophilic	~43 kDa	12	Collectin	innate pulmonary immune response	~1 %

1.4 LUNG SURFACTANT PROTEINS A AND D

SP-A and SP-D belong to the collectin (collagenous lectin, i.e. carbohydrate-binding proteins) family and are expressed in the lung, primarily in the alveolar type II cells but also in upper airways and so called Clara cells and in other organs and tissues like the gastric and intestinal mucosae (37, 38). SP-A and SP-D may have several different functions in lung surfactant, SP-A for example binds to the crossings in TM. However, the main function of both SP-A and SPD is in lung immunity, i.e. to take part in the defense against invading pathogens. SP-A and SP-D are comparatively big water-soluble proteins that can bind to pathogens through their C-terminal carbohydrate recognition domains (CRD) that form globular head groups (39, 40). Both the pulmonary collectins are oligomers formed by joining several trimeric units together; SP-A is composed of six trimers, i.e. totally 18 subunits of ~35 kDa each (41, 42), while SP-D is built up by four trimers, i.e. totally 12 subunits of ~43 kDa each (43, 44). The SP-A and SP-D activities in the innate pulmonary immune response are manifold; they stimulate chemotaxis, promote phagocytosis and contribute to aggregation and opsonization of pathogens (45). SP-A has also been shown to participate in the uptake and secretion of surfactant from alveolar type II cells, as well as in the formation of TM, while SP-D has shown to contribute to the surfactant homeostasis (46, 47). SP-D deficiency is associated to an increased concentration of surfactant in lung cells and the alveolar space (48, 49). Neither SP-A nor SP-D apparently has any direct role in reducing surface tension in the alveoli (50), which is indirectly supported by the fact that they are not present in natural derived surfactant preparations (29, 51). The surfactant preparations derived from animal origins used for exogenous replacement therapy are obtained by one or several purification steps in organic solvents, which results in that the hydrophilic SP-A and SP-D are efficiently removed, while phospholipids, SP-B and SP-C remain soluble.

Therefore SP-B and SP-C are the target proteins for development of synthetic lung surfactant preparations and the main focus of the present thesis.

1.5 Lung surfactant proteins B and C

SP-B and SP-C are small lipid-associated polypeptides that are important for lowering the surface tension in the alveoli (51). SP-B and SP-C are synthesized in the type II alveolar cells from different precursor proteins that go through multiple proteolytic cleavages and other posttranslational modifications as they traverse the Golgi apparatus, MVB and LB (52-55). The above facts as regards overall properties of SP-B and SP-C may suggest that they also are structurally and functionally similar. This is however not the case: SP-B and SP-C are structurally completely unrelated, and they apparently perform different functions in lung surfactant (56-62).

1.5.1 Surfactant protein B

The native SP-B molecule consists of a polypeptide chain with 79 amino acid residues, that is covalently linked into a homodimer with a total molecular weight of 17.4 kDa (58, 63). The primary structure of SP-B is characterized by the presence of seven cysteines, six of which form three intra-molecular disulfide bridges (Cys8 to Cys77, Cys11 to Cys71 and Cys35 to Cys46) and Cys48 that forms an inter-molecular disulfide bridge with a second subunit, resulting in the covalently linked homodimer (60, 64). The tertiary structure of SP-B has not been experimentally resolved, however it is known that it has a high α -helical content in lipid environments and in organic solvents, as determined using circular dichroism (CD) and Fourier transform infrared (FTIR) spectroscopy (65-69). SP-B belongs to the saposin-like protein (SAPLIP) superfamily and the structural homology between proteins from this family combined with experimental structural studies of other SAPLIP members, have given some important insights into the SP-B structure. Modeling studies using NK-lysin (70) and saposin B (71), for which there are experimentally determined NMR and X-ray structures, respectively, suggested that the SP-B structure contains 4-5 α -helices folded into a V-shape arrangement with helices 1 and 5 on one side, helices 2 and 3 on the other side and helix 4 in between (Figure 3). It should be pointed out that SP-B differs in some aspects from the other SAPLIP members: it is a dimer, soluble in organic solvents, whereas the other members are water-soluble monomeric proteins. This makes modeling of SP-B based on other SAPLIP structures difficult and all models proposed should be interpreted with caution. The covalent disulfide-linked

nature of SP-B puts some constraints on what parts of the two subunits are close, and based on this the SP-B homodimer has been suggested to be promoted by salt bridges at the subunit interface, created between the charged residues Glu51 and Arg52 from different subunits (70).

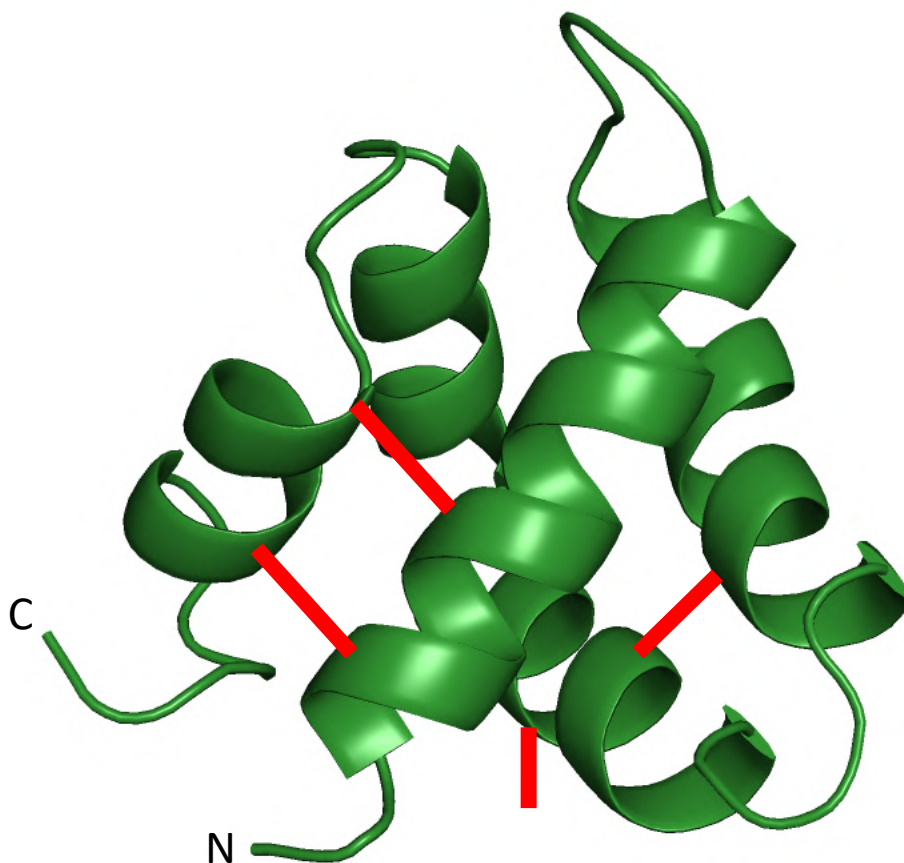


Figure 3. Hypothetical structural model of monomeric SP-B. Cartoon representation of the polypeptide backbone structure of the SP-B homologous protein NK-lysin from pig. The hypothetical intra- and inter-molecular disulfide bonds are represented with red lines. N and C indicate the N- and C- terminus of the peptide respectively. PDB code 1NKL (72).

In native SP-B, the net charge of +14 (9 positively and 2 negatively charged residues per subunit) and the about 60% of the amino acid residues with strictly hydrophobic side chains are likely important for its strong interaction with lung surfactant phospholipids (73). In spite of the overall hydrophobic nature and requirement of organic solvents for solubilization, SP-B attaches only peripherally to bilayers of phospholipids and lacks transmembrane regions (74, 75). The detailed molecular mechanisms of SP-B function in pulmonary surfactant are still

unknown. However, the dimeric nature and ability of SP-B to interact with the outer parts of phospholipid membranes can make it able to cross-link two different phospholipid layers (62, 70, 76, 77). Such interactions of SP-B could promote dense phospholipid bilayer packing, induce fusion and lysis of phospholipid layers as well as mediate transport of phospholipids between membranes. Dense packing of layers of pulmonary surfactant phospholipids, possibly mediated by SP-B, can be beneficial in formation of the densely packed LB and also help to stabilize different phospholipid layers at the end of expiration (62, 78). The ability to fuse or lyse phospholipid layers has been observed for isolated SP-B segments (79) and under physiological conditions, such actions of SP-B could promote fast adsorption of phospholipids to the air/water interphase during dynamic respiratory cycling (34, 80). It has also been suggested that SP-B dimers can associate to form larger oligomers with a ring-shape, which could promote the transport of phospholipids between layers (71).

ProSP-B is the precursor protein of SP-B, which undergoes glycosylation outside the SP-B region. ProSP-B consists of 381 residues, has a molecular weight of 42 kDa and is synthesized into the ER, directed by the presence of a signal peptide (63). The subsequent maturation process occurs in the Golgi apparatus and possibly in the MVB and it is mediated by at least three different proteolytic enzymes: napsin A, cathepsin H, and pepsinogen C (53, 81). The details of the proteolytic maturation are still uncertain. Mutations in the *SFTPB* gene (that encodes proSP-B) in humans as well as in mouse models are associated with SP-B deficiency, which causes lethal RDS in the neonatal period (82-84). SP-B deficiency is linked to an aberrant processing of proSP-C (see further below) that results in formation of an inactive, misprocessed SP-C variant that is secreted into the extracellular alveolar space and accumulates in the alveoli (85, 86). Lung transplantation is currently the only available treatment for SP-B deficiency caused by *SFTPB* mutations (26).

1.5.2 Surfactant protein C

SP-C is a 35 amino acid residue lipopeptide and has a molecular weight of 4.2 kDa (59, 61). It is a cationic peptide with a net charge of +3 and it is probably the most hydrophobic protein so far described in mammals since it consists of about 80 % amino acid residues with hydrophobic side chains and it contains one or two (depending on animal species of origin) covalently attached fatty acyl chains to Cys5 and/or Cys6 via thioester bonds (87, 88). SP-C does not belong to any known protein family and is exclusively expressed by the alveolar type II cells. The tertiary structure of porcine SP-C dissolved in organic solvents was described in 1994 and

shows one long α -helix that makes up the C-terminal two thirds of the peptide and a structurally disordered N-terminal part (89) (Figure 4). More specifically, the α -helix is composed of residues 9-34 and its length of 37Å perfectly matches the thickness of a fluid DPPC bilayer (90). Also, the length of the helix segment that contains only non-polar residues matches the thickness of the hydrophobic fatty acyl part of a DPPC phospholipid bilayer (90). These correlations strongly suggest that SP-C is inserted in a transmembrane orientation in a phospholipid (DPPC) bilayer, whereby the non-polar residues of the α -helix are positioned to interact with the hydrocarbon chains of the phospholipids, and the positively charged residues Lys11 and Arg12 are suitably located to interact with the polar phospholipid head groups (90). It has been suggested that SP-C could alternatively interact with phospholipids in a monolayer, like the monolayer supposedly located at the alveolar air/water interface, by adopting a tilted position of about 70° (91).

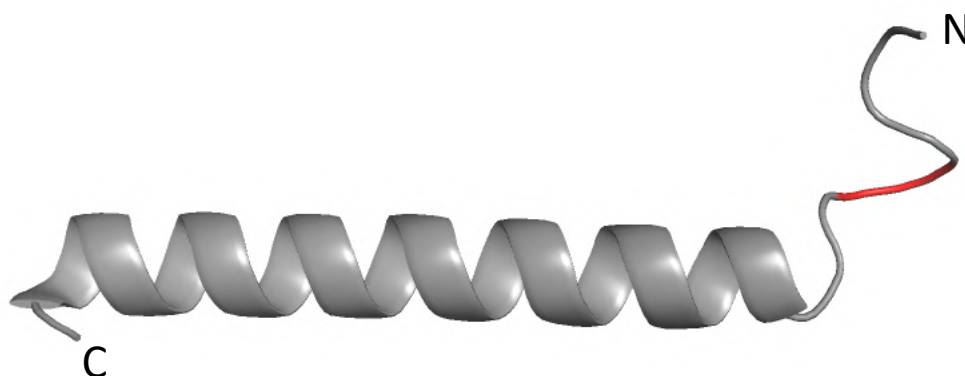


Figure 4. Human SP-C structure. Three-dimensional structure of the polypeptide backbone of human SP-C determined in a mixed solvent containing $C_2H_5Cl/C_2H_5OH/1\text{ M HCl}$ 32:64:5 (by volume). The portion of the structure marked in red represents the location of the cysteines that are palmitoylated in native SP-C. N and C indicate the N- and C- terminus of the peptide respectively. PDB code: 1SPF (89).

The α -helix of SP-C contains a high number of valines that are prone to form β -sheet aggregates *in vitro* resulting in amyloid-like fibrils (92, 93). The dominance of Val residues in the SP-C α -helix is unexpected, since Val has the highest β -strand propensity of all 20 naturally occurring gene-encoded residues (92, 93). This feature is unique to SP-C and the reason why its α -helix contains a poly-valine region is unknown, however several possible explanations

have been suggested. The poly-valine α -helix confers a very stable and rigid structure that could be used to promote rapid adsorption by perturbing the phospholipid layers (90, 94). Another possible explanation is that amyloid fibrils are formed under certain conditions, promoted by the high β -strand propensity of Val and that they play a physiological role, for example, in the defense against bacteria or viruses (95), in a similar manner as certain defensins can form fibrillar nets in the gastrointestinal tract in the defense against bacteria (96, 97).

The precursor protein of SP-C, proSP-C contains a BRICHOS domain that prevents the aggregation and misfolding of the SP-C part during biosynthesis (98-100). The BRICHOS domain is able to bind nonhelical poly-valine segments and to promote their folding into helical conformations (98, 101, 102). In addition to its physiological target peptide, the BRICHOS domain has turned out to be able to prevent amyloid formation also of other proteins, for example amyloid- β (A β) and islet amyloid polypeptide (IAPP) (103-107). These features make the BRICHOS domain an interesting candidate for development of therapeutic strategies against amyloid diseases, in particular the BRICHOS domain from the Bri2 protein seems to have properties that can be harnessed against neurodegenerative diseases (103, 108, 109).

ProSP-C is a 21 kDa protein of 197 amino acid residues, cotranslationally inserted into the ER membrane and transported to the Golgi apparatus where it is palmitoylated (110). During the maturation process, proSP-C is cleaved by several enzymes (111-113), eventually releasing the mature form of SP-C, which like SP-B ends up in the extracellular alveolar space after secretion together with phospholipids in the LB. Again, although SP-B and SP-C are overall similar in being secreted together with phospholipids, the details differ. ProSP-C gets membrane-inserted already into the ER, where the SP-C part acts as a signal peptide and is then transported and processed all the way to the alveoli as an integral membrane protein (110, 114). ProSP-B, in contrast, is translocated into the ER lumen, directed by a traditional, N-terminally located signal peptide that is removed after translocation to the ER lumen, and the proteolytically released SP-B part becomes associated with surfactant membranes at a later stage, probably in the MVB (52, 115, 116).

Mutations in the gene encoding proSP-C, *SFTPC*, are more common than mutations in *SFTPB* and most of them are located in the BRICHOS domain (100, 117). *SFTPC* mutations result in lack, or reduced levels, of mature SP-C and also result in aberrant protein folding and (pro)SP-C aggregation and amyloid fibril formation. Misfolding of (pro)SP-C associated with *SFTPC* mutations results in disease, with different degrees of pathology, from mild interstitial lung disease (ILD) to severe pulmonary fibrosis (100, 118-122). Notably, mutations in *SFTPC*, in

particular those that affect the BRICHOS domain, result in amyloid disease in early infancy, in contrast to other amyloid diseases, which do not affect humans until late life. This attests to the extreme amyloidogenic potential of the (pro)SP-C poly-Val segment. Interestingly, pulmonary surfactant from birds does not contain SP-C and is less effective in lowering the surface tension *in vitro* than surfactant from mammals. Bird lungs have a different physiology and morphology compared to mammalian lungs. Therefore, the function of bird surfactant is not to prevent alveoli from collapsing but to keep the air capillaries open (123).

1.6 Neonatal and acute respiratory distress syndrome

Respiratory distress syndrome (RDS) in neonates is characterized by insufficient amounts of pulmonary surfactant and results in respiratory failure. The lungs of more than about 10 weeks prematurely born infants are simply not prepared to breathe air and have an underdeveloped surfactant system. The lack of pulmonary surfactant is associated with alveolar collapse and with concomitant difficulties in oxygenation and damage to the alveolar epithelium including the surfactant producing type II cells, which results in a vicious cycle (124). The by far most common reason for the lack of sufficient pulmonary surfactant in prematurely born neonates is that their immature lungs are unable to produce sufficient amounts of pulmonary surfactant. Genetic defects can also affect pulmonary surfactant function, and mutations in the genes encoding proSP-B (*SFTPB*) or ABCA3, a phospholipid transporter present in LB can give rise to neonatal RDS in full term infants (125). RDS is a life-threatening disease and the development of surfactant replacement therapy with natural derived surfactant preparations in the 1980s have saved millions of lives (95).

Another condition associated with pulmonary surfactant dysfunction is acute respiratory distress syndrome (ARDS), also referred to as acute lung injury (ALI). The pathogenesis behind reduction of pulmonary surfactant activity in ARDS, where surfactant inhibition is a prime cause, differs qualitatively from that in neonatal RDS, where surfactant deficiency is the main underlying factor (Table 2). ARDS can be elicited by several different conditions, including direct lung injury, for example pneumonia or aspiration of toxic substances, or be a result of severe systemic diseases, for example sepsis or multitrauma (126). ARDS can occur in individuals of all ages and is associated with high mortality, up to 40%, the exact risk depending on the severity and nature of the underlying condition (127, 128). ARDS is characterized by bilateral pulmonary infiltrates and arterial hypoxemia identified when the partial pressure of arterial oxygen (PaO_2) divided by the inspired fraction of oxygen (FiO_2) is

equal to or less than 300 mmHg ($\text{PaO}_2/\text{FiO}_2 \leq 300 \text{ mmHg}$) (129). The infiltrates and hypoxia are a result of a pulmonary edema that releases blood proteins and other molecules in the alveolar space, altering the gas exchange and reducing the pulmonary surfactant activity (130). The pathology of ARDS is more complicated than the surfactant deficiency in RDS and despite the study of numerous therapies to treat ARDS (surfactants, glucocorticoids, antioxidants) there is still no specific treatment (131-134). The surfactant activity in ARDS can be impaired by reducing the adsorption of the surfactant to the air/water interface (by blood proteins), changing the composition of the surfactant (by fatty acids and phospholipids) and/or chemically altering the phospholipids and proteins of the surfactant (by phospholipases, proteases and reactive oxygen and nitrogen species) (135-138). The development of a surfactant preparation that is more resistant to inactivation and increasing the concentration of active pulmonary surfactant by instilling high amounts of exogenous surfactant in the patient could contribute to the development of a successful therapy for ARDS.

Table 2. Prominent features of neonatal and acute RDS.

	Neonatal RDS	Acute RDS (ARDS)
Age of patient	Neonate	Any age
Characterized by	Insufficient pulmonary surfactant	Inactivation of pulmonary surfactant
Causes	Prematurity of the lungs or mutations in precursors of surfactant proteins or proteins related to the surfactant production	Direct lung injury (for example: pneumonia or aspiration of toxic substances) or severe systemic diseases: (for example: sepsis or multitrauma)
Treatment	Surfactant replacement therapy	Supportive care and protective ventilation Treatment of comorbidities

1.7 Surfactant replacement therapy

The most effective and only commercially available drug treatment for neonatal RDS is surfactant replacement therapy, using natural derived surfactant preparations from bovine or porcine lungs. These preparations contain phospholipids and the hydrophobic surfactant proteins SP-B and SP-C (29, 51).

Today different natural derived surfactants are approved. In USA five surfactant preparations have FDA approval but the two synthetic surfactants colfosceril palmitate (Exosurf®) and Lucinactant (Surfaxin®) are not commercially available any longer in contrast to the three

natural derived surfactants: beractant (Survanta®), calfactant (Infasurf®), and poractant alfa (Curosurf®) (Table 3). In other countries bovactant (Alveofact®) and/or surfactant-TA (Surfacten®) are available.

Table 3. Composition of natural derived surfactants approved by FDA

	Poractat alfa	Beractant	Calfactant
Commercial name	Curosurf®	Survanta®	Infasurf®
Preparation	Minced porcine lung extract	Minced bovine lung extract	Bovine lung lavage
PL (mg/ml)	76	25	35
Disaturated PC (mg/ml)	30	11-15.5	16
Total proteins (mg/ml)	1	0.1-1	0.65
SP-B (mg/ml)	0.45	Not specified	0.26
SP-C (mg/ml)	0.59	Not specified	0.39
Dose (mg/kg)	100-200	100	100
Volume	1.25-2.5 ml/kg	4 ml/kg	3 ml/kg
FDA approval	1999	1991	1998

PL, phospholipid; FDA, Food and Drug Administration

As described above, the natural derived surfactants provide an effective therapy of neonatal RDS. However, their production is costly, there is a limited supply, the rather crude preparation methods may give batch to batch variability, and there is a theoretical risk of transmitting infectious diseases. These drawbacks motivate the attempts to complement or replace the natural derived surfactant preparations with fully synthetic alternatives, which have been ongoing since the 1980s. Nevertheless, the development of synthetic surfactant alternatives that are at least as effective as the natural surfactants and that can be produced at low cost has turned out to be a significant challenge (95).

Surfactant replacement therapy with natural derived surfactants to treat ARDS, although attractive from a theoretical point of view, has been inefficient in clinical trials, mostly due to surfactant inactivation (131). Synthetic surfactants that could be efficiently produced at a large scale, and possibly made more resistant to inactivation than the natural derived counterparts

are therefore good candidates for the development of a surfactant preparation that can be used to treat ARDS patients (139-141).

1.8 Synthetic lung surfactant protein analogues

Endogenous surfactant has a very complex and diverse composition that is difficult to replicate faithfully in synthetic preparations. The first synthetic surfactant preparations developed contained only phospholipids, primarily a large fraction of DPPC and they were not efficient, because DPPC has a high melting temperature (41°C) and does not spread efficiently under physiological conditions (142). An active surfactant with good surface activity and efficient spreading properties therefore needs to combine DPPC with other phospholipids and with proteins in order to lower the melting temperature and promote spreading. Much effort has been put into finding a simple phospholipid composition that can be used to produce synthetic pulmonary surfactants, but still there is no consensus on what is the optimal composition (143, 144). In addition, as described above, SP-B and SP-C are structurally and likely also functionally distinct, and in line with this notion both proteins are necessary for the optimal function of reconstituted surfactant preparations in animal models of RDS (56, 57). However, native SP-B and SP-C are difficult to produce due to several factors, including complex intra- and intermolecular disulfide linkages, strict hydrophobicity, posttranslational modifications in the form of thioester-linked fatty acyl chains, and a pronounced tendency to aggregate. In fact, with the exception of recombinant SP-C (145), production of functional SP-B, independent of production method (recombinant or synthetic), or synthetic SP-C has not been reported to date. Attempts have been made, but it was for example shown by fluorescence anisotropy that native SP-B and synthetically produced full length SP-B interact differently with lipid bilayer surfaces (146). For these reasons, the development of functional SP-B and SP-C analogues that can be produced in a robust manner is needed.

1.8.1 SP-B analogues

Attempts to produce recombinant full length SP-B fused to urokinase (147) or in the proprotein form (148) have been made, however the produced proteins' abilities to reduce surface tension has to our knowledge not been published. Given the structural complexity of native SP-B, development of SP-B analogues has to a large extent aimed to reproduce its function but using less complex peptide designs. The studies of small linear peptide fragments of SP-B have helped to understand the structure-function relationships of individual regions of the SP-B polypeptide chain. The fragment corresponding to residues 7-22 that comprise the first helix

induce liposome lysis. A longer fragment, corresponding to residues 1-22, was shown to promote lipid mixing, but only when the peptide included also parts corresponding to helix 2 (residues 1-37) it was able to induce liposome fusion. Neither the C-terminal fragment (residues 43-79) nor the synthetic full length SP-B were able to enhance the fusogenic activity shown by the N-terminal half alone (79). From these results it was concluded that the N-terminal half of SP-B is the main responsible region for SP-B ability to fuse and lyse phospholipid membranes, and also to reduce surface tension *in vitro*. Another study showed that dimerization of the SP-B fragment containing residues 1-25, mediated by a disulfide bridge, resulted in a peptide that, when mixed with phospholipids, was more efficient in reducing surface tension *in vitro* than the monomeric form, and it was comparable *in vitro* to native SP-B (149). The results of this study support the concept that SP-B function relies to some extent on a homodimeric nature and also points to the importance of the presence of at least two helices in SP-B analogues.

Walther and Waring designed a 34-residue SP-B analogue called Mini-B, which is composed of a linear polypeptide corresponding to residues 8-25 and 63-78 of native SP-B (150). These two polypeptide segments correspond to the first and last predicted helices of SP-B, which are overall hydrophobic but contain 4 and 3 positive charges, respectively. The tertiary structure of Mini-B in sodium dodecyl sulfate (SDS) micelles determined by NMR spectroscopy shows that the two predicted helices indeed form a helix-loop-helix hairpin stabilized by two intramolecular disulfide bonds (corresponding to Cys8-Cys77 and Cys11-Cys71 in native SP-B) (151) (Figure 5). Synthetic surfactant created by combining phospholipids and Mini-B showed comparable activity *in vitro* and better performance in a rat model of surfactant deficiency than a mixture of phospholipids and native SP-B (150). Mini-BLeu is an SP-B analogue that is similar to Mini-B except that two methionines have been substituted by leucines to promote hydrophobicity and to avoid unintentional oxidation of the methionines (143).

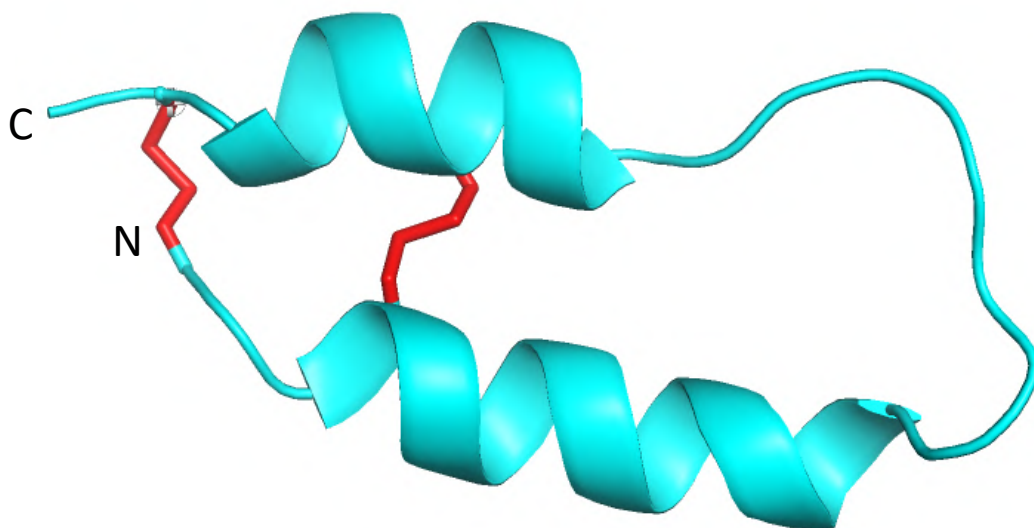


Figure 5. Three-dimensional structure of the SP-B analogue Mini-B. Cartoon representation of the polypeptide backbone of Mini-B where the disulfide bonds are marked in red. N and C indicate the N- and C- terminus of the peptide respectively. PDB code: 2DWF (151).

Later studies suggested an important role for the N-terminal 7 residues of SP-B, which are not included in Mini-B (152). The analogue formed when these 7 residues were linked to Mini-B in one polypeptide was referred to as Super Mini-B (141). The 7-residue N-terminal motif is very hydrophobic, and its presence in Super Mini-B, or native SP-B, could thus favor interactions with phospholipid membranes of synthetic and native pulmonary surfactant, respectively. CD spectroscopy of a peptide corresponding to only the 7 N-terminal residues of SP-B suggest a lack of α -helical structure and the possibility of random coil or β -sheet structures. This correlates with the presence of three prolines that can prevent helix formation. Moreover, Super Mini-B has an α -helical content observed by CD spectroscopy that is similar to that of Mini-B (152), a result which further supports that the N-terminal seven residues lack stable secondary structure also when they are part of Super Mini-B. At submicellar concentrations of SDS Super Mini-B is a more stable non-covalent dimer than Mini-B, and it thus more resembles the dimeric structure of native SP-B (141). In addition, Super Mini B mixtures with phospholipids have been shown to be more active than Mini-B mixtures *in vitro* and *in vivo* (140, 141).

In order to create a more stable and, in particular easier to manufacture SP-B analogue, the analogue B-YL was produced, in which the four Cys of Super Mini B were substituted by Tyr and the two Met were replaced by Leu. B-YL showed good surface activity *in vitro* and the

mixture of B-YL with synthetic phospholipids resulted in similar oxygenation and compliance as Curosurf[®] in a surfactant deficiency adult rabbit model (153).

A different type of analogue designed based on SP-B is called KL4. KL4 is a linear peptide formed by the pentapeptide segment LysLeuLeuLeuLeu repeated four times and ending with a Lys (Table 4). This results in a 21-residue peptide that is rather straightforward to synthesize due to its lack of Cys and that forms an α -helix (154). The KL4 design was reportedly based on features of the C-terminal part of SP-B and it was supposed to interact with superficial parts of phospholipid membranes, like SP-B (154). This hypothesis has been questioned based on results that showed that the KL4 helix is localized in a transmembrane manner in phospholipid bilayers, thus more resembling SP-C (155, 156). Synthetic surfactant preparations based on KL4 have shown promising results *in vitro* and *in vivo* (157-160). A synthetic surfactant preparation, called Lucinactant (Surfaxin[®]), containing KL4 mixed with mainly synthetic phospholipids but also palmitic acid was approved by the US Federal Drug Administration in 2012 for prevention of RDS in premature infants, after conduction of successful clinical trials (161). However, the company ceased with the production of Surfaxin[®] as part of a restructuring plan.

Several other molecules have been reported to behave as functional analogues of SP-B, for example polymyxin E (70) and peptoids (162). These analogues are not further described here since our studies have been focused on conventional L-amino acid peptide-based analogues, which have the advantage that they can be produced by recombinant methods.

Table 4. Comparison of amino acid sequences of human SP-B and SP-B analogues. The sequence differences of the SP-B analogues from the human SP-B are marked in red.

Polypeptide	Sequence
Human SP-B	FPIPLPYCWLCRALIKRIQAMIPK GALAVAVAQVCRVPLVAGGICQCLAERYSVILDDTLGRMLPQLVCRLVLRCSM
Mini-B	-----CWLCRALIKRIQAMIPK-----GRMLPQLVCRLVLRCS-----
Mini-BLeu	-----CWLCRALIKRIQALIPK-----GRLLPQLVCRLVLRCS-----
Super Mini-B	FPIPLPYCWLCRALIKRIQAMIPK-----GRMLPQLVCRLVLRCS-----
B-YL	FPIPLPYWLYRALIKRIQALIPK-----GRLLPQLVYRLVLRYS-----
KL4	-----KLLLKLKLLKLLKLLKLLKLLK-----

1.8.2 SP-C analogues

Native SP-C is difficult to obtain both by chemical synthesis and by recombinant production due to the pronounced propensity of the poly-valine segment to form β -sheet aggregates instead of the native α -helix. However, it has been shown that production of recombinant human SP-C with the two palmitoylated Cys substituted by Phe can result in a helical peptide (163). A synthetic surfactant called Lusupultide (Venticute[®]) containing this recombinant version of SP-C, rSP-C and a synthetic phospholipid mixture improved lung function in animal models (163-166), and was tested in clinical trials for efficacy against ARDS, but turned out not to show significant positive effects (139, 167). A lot of effort has likewise been put into designing active SP-C analogues that avoid β -sheet formation, by modifying the poly-valine sequence. After observing a high helical content, no aggregation and a good *in vitro* surface activity of an SP-C analogue where the entire poly-valine region was substituted by the second transmembrane helix of bacteriorhodopsin, it was suggested that an α -helical transmembrane conformation is important for the biological function of SP-C, while the exact amino acid sequence of the transmembrane helix is of less importance (168).

Based on the results with the SP-C/bacteriorhodopsin hybrid, another analogue called SP-C(Leu) was created by substituting every helical valine by leucine (that favors α -helix conformation), and replacing the palmitoylated cysteines by serines (to avoid unwanted disulfide bridges in the absence of palmitoyl-Cys) (Table 5). A method for chemical acylation of SP-C analogues has been developed (169) but palmitoylated SP-C(Leu) did not show better activity than the non-acylated peptide in a rabbit model of RDS (144). For this reason and the added complexity of synthesizing acylated SP-C analogues, further analogues all have had Ser instead of palmitoyl-Cys.

SP-C(Leu) was shown to have an α -helical content and surface-active properties *in vitro* similar to those of native SP-C. It was also shown that a mixture of synthetic phospholipids and 2% SP-C(Leu) improved lung dynamics in preterm rabbit fetuses, an RDS model, in comparison to non-treated controls. However, SP-C(Leu) can form helical oligomers, a property that differs compared to the mainly monomeric form of native SP-C under the same conditions (170). It has indeed been shown that SP-C with a poly-valine sequence can form a dimer, mediated by a motif in the C-terminal part (95, 145) but higher order oligomers as observed for SP-C(Leu) are likely less functional than monomers. In order to avoid oligomerization of SP-C(Leu), three lysines spaced with four residues were introduced. This analogue was called SP-CLKS and it

was shown to promote phospholipid adsorption to an air/water interface *in vitro* (171). However, SP-CLKS did not improve lung function in the premature rabbit RDS model (144) and therefore yet another analogue with only one added lysine in the beginning of the poly-leucine region, deletion of the two N-terminal residues (resulting in a 33-residue peptide) was designed, referred to as SP-C33. SP-C33 did not show oligomerization and in mixture with phospholipids it was as active as the natural derived surfactant Curosurf® in a premature rabbit model of RDS, provided that a positive end expiratory pressure (PEEP) was applied during artificial ventilation (144). Later on, it was shown that in the absence of PEEP, surfactants containing both SP-C33 and native SP-B, or Mini-B, were more active in an animal model of neonatal RDS than surfactant preparations containing only SP-C33 or only SP-B (56, 57). Additional peptide and peptoid analogues of SP-C and SP-C(Leu) have been designed and analyzed, but for the same reason as for non-peptide SP-B analogues they are not described in here. A recent review of the development of synthetic surfactant preparations describe these analogues in detail (95).

Finally, SP-C33Leu (Figure 6) is an SP-C analogue similar to SP-C33 but the methionine in position 31 has been substituted by leucine, in an analogous manner as Mini-BLeu was created to avoid unintentional oxidation. SP-C33Leu is structurally similar to SP-C33 and it has been shown to be as active as Curosurf® *in vitro* and *in vivo* (172-174).

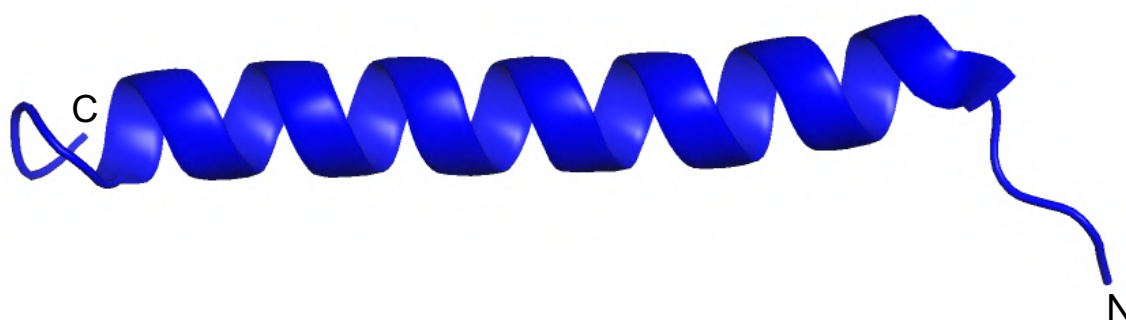


Figure 6. Three-dimensional structure of the SP-C analogue SP-C33Leu determined in organic solvents. Cartoon representation of the polypeptide backbone. N and C indicate the N- and C- terminus of the peptide respectively. PDB code: 5NDA (172).

Table 5. Comparison of amino acid sequences of human SP-C and SP-C analogues. The two Cys in yellow in human SP-C are palmitoylated. The sequence differences of the SP-C analogues from the human SP-C are marked in red.

Polypeptide	Sequence			
	1	10	20	30
Human SP-C	FGIP	CCPVHLKRL	LIVVVVVV	LIVVVIVGALIMGL
rSP-C	FGIP	FFPVHLKRL	LIVVVVVV	LIVVVIVGALIMGL
SP-C/bacteriorhodopsin	FGIPCC	PVHLKR	FYAITTLVAA	I AFTLYLSLLGY
SP-C(Leu)	FGIP	SSPV-LKRL	LI LLLLLL	LI LLLI LGALIMGL
SP-CLKS	FGIP	SSPVHLKRL	LI LKLLLLL	KL LLLLKL GALIMGL
SP-C33	--IP	SSPVHLKRL	KLLLLLLLL	LI LLLI LGALIMGL
SP-C33Leu	--IP	SSPVHLKRL	KLLLLLLLL	LI LLLI LGALLGL

1.9 Synthetic lung surfactants

As described above several lung surfactants containing synthetic phospholipids and different single peptides, for example, recombinant SP-C, Mini-B, Super Mini-B, SP-C(Leu), or SP-C33 have been created over the years. These surfactants have overall shown good results *in vitro* and *in vivo* and it has been shown that synthetic surfactants containing analogues of both SP-B and SP-C are more efficient than surfactants with only one peptide analogue (56, 143, 175).

A synthetic surfactant preparation called CHF5633, containing both Mini-BLeu (0.2%) and SP-C33Leu (1.5%) mixed with DPPC:palmitoyl-oleyl-phosphatidylglycerol (POPG) (1:1, w/w)) is now in clinical trials (174). CHF5633 is as good, or better than animal-derived surfactants in rabbit and lamb models of preterm RDS or surfactant inhibition, respectively (173, 176).

The cost and therefore the availability of a synthetic surfactant can potentially be reduced if the peptide analogues are produced recombinantly instead of by chemical synthesis. In addition,

chemical synthesis can result in truncated versions of the peptide or other by-products that can be difficult to remove. Therefore, recombinant production of SP-B and SP-C analogues is an attractive strategy, which we have explored in papers **II**, **III** and **IV** of this thesis.

2 THIS STUDY

2.1 Aims

The main and overall aim of this thesis was to develop surfactant protein analogues that combined with simple phospholipid mixtures can be used to treat neonatal RDS and ARDS in animal models, with at least the same efficiency as natural derived surfactant preparations.

The four individual projects of this thesis were based on the following more specific aims:

- to design SP-B analogues with different intramolecular disulfide patterns and study the effects of mixtures of each of these analogues with an SP-C analogue and phospholipids in a rabbit fetus model of neonatal RDS (**Paper I**).
- to optimize the purification process of recombinant SP-C33Leu, mix the purified peptide with synthetic phospholipids to obtain a cost-efficient synthetic pulmonary surfactant (rSP-C33-surfactant) (**Paper II**), and to evaluate it in animal models of neonatal RDS (**Paper II**) and ARDS (**Paper III**).
- to design, produce, purify and analyse novel analogues that potentially combine the properties of SP-B and SP-C in one polypeptide chain and to investigate if mixtures of these Combo peptides with phospholipids are functional in a rabbit model of neonatal RDS, even in the absence of lung-stabilising ventilation with PEEP (**Paper IV**).

2.2 Methods

This section is a summary of the salient features of the more unconventional methods used in papers **I-IV**. For a more complete description see the material and methods sections of the respective papers.

Modified N-terminal domain (NT) from spidroins as solubility tag for recombinant protein production

The target proteins produced recombinantly in papers **II**, **III** and **IV** have been expressed as fusion proteins using solubility tags to enhance their solubility and avoid aggregation during expression. The solubility tags used were NT* (also called NT*_{MaSp}) in papers **II** and **III** and FLSpNT* (also called NT*_{FISp}) in paper **IV**. These tags are based on the N-terminal domain (NT) of two different spider silk proteins (spidroins) developed by our group, from the idea that NT works as a solubility enhancer that keeps the aggregation-prone spidroins in solution during storage in the silk gland (172, 177). During spider silk formation NT dimerizes (178, 179) and in order to avoid dimerization NT* in which Asp40 was substituted with Lys and Lys65 with Asp was developed (172, 180, 181). In addition to being a constitutive monomer, NT* is very stable and hypersoluble, and can successfully be used to produce recombinant, active SP-C33Leu (rSP-C33Leu) in *E. coli* (172).

Alkaline hydrolysis and two-phase separation

For purification of peptides, the bacterial lysates obtained after recombinant production in paper **IV**, were subjected to alkaline hydrolysis using 0.2 M KOH at 40°C for 60 min. The ester bonds in the lipids (see Scheme 1) are cleaved forming above all free fatty acids, glycerol, and phosphoric acid. Following the hydrolysis, a two-phase separation was performed using chloroform/methanol/water 8:4:3 (by volume). The lower phase was washed by removing the upper phase and adding more methanol/water 1:1 to create a new upper phase. The upper phase contained most of the hydrolyzed lipids while the peptide stayed in the lower organic phase.

Lipidex-5000 chromatography

Reverse phase Lipidex-5000 liquid chromatography was used in papers **II**, **III** and **IV** in order to separate the target peptides from remaining lipids. Samples resuspended in methanol:dichloroethane:water, 85:10:5 (by volume) in papers **II** and **III** and in methanol/ethylene chloride/0.1 M HCl, 85:10:5 (by volume) in paper **IV** were applied to a first Lipidex-5000 column. The first column volume was collected, dried, resuspended in the same solvent and subjected to separation with higher resolution using a longer Lipidex-5000 column than the first one. The fractions containing the target peptide were selected from the appearance of Coomassie stained bands after SDS-PAGE. In the reverse phase Lipidex-5000 column the polypeptides are expected to elute early followed by the phospholipids and finally by neutral lipids. The use of an acidic mobile phase in paper **IV** was intended to acidify the peptide and therefore reduce the risk of aggregation.

Sephadex LH 60 chromatography

In paper **IV**, the components obtained after the Lipidex-5000 chromatography gave rise to two bands in the same fractions, observed by SDS-PAGE, that were possible to separate in the Sephadex LH 60 column. Sephadex LH 60 is a size exclusion lipophilic column that can be used to separate lipophilic molecules in organic solvents, for example, lipids and small peptides (51). The sample (that contained two bands) obtained from the second column of Lipidex-5000 in the purification of Combo peptide 6 in paper **IV** was dried, resuspended in chloroform/methanol/0.1 HCl, 19:19:2 (by volume) and applied to a Sephadex LH 60 column. Combo peptide 6 was identified as the upper band on SDS-PAGE and eluting first on Sephadex LH 60 chromatography, by using N-terminal sequence analysis by Edman degradation.

Synthetic surfactant mixtures

Different percentages (0.75-8%, by weight) of the peptides obtained after chemical synthesis in papers **I** and **IV** and recombinant expression and purification in papers **II**, **III** and **IV** were mixed with phospholipids to create different synthetic surfactants. We used two different phospholipid compositions: DPPC/egg yolk-PC/POPG, 50:40:10 (by weight) that has been shown to be effective in previous studies (143) and DPPC/POPG, 50:50 (by weight) that is used in the synthetic surfactant CHF5633 (173). Dried phospholipids and dried peptides were resuspended in chloroform/methanol, 2:1 (by volume) and mixed. After drying the samples

carefully under reduced pressure, they were resuspended in physiological saline (154 mM NaCl in water) to 80 mg/ml of phospholipids.

RDS rabbit model

The RDS premature rabbit fetus model essentially as introduced by Enhörning and Robertson (8) was used in papers **I**, **II** and **IV**. This model was used to test the mechanical properties of the lungs of premature rabbit fetuses with RDS after tracheal instillation of different pulmonary surfactant preparations (Figure 7). The animals were ventilated after the instillation of surfactant, with the help of a ventilator using high pressures, which creates tidal volumes that are much higher than the physiological values. This is a good screening model to evaluate the *in vivo* activity of exogenously delivered surfactant. Animals that are not instilled with any surfactant mixture are used as negative controls and should have very low tidal volumes and thus low lung compliance, to verify the immaturity of the litter. The lung gas volumes are a quantification of the residual gas inside the lungs after the end of mechanical ventilation, and are measured using a water displacement technique (182). The studies to test the surfactant preparations containing phospholipids only or phospholipids and the SP-C analogue rSP-C33Leu (i.e. no SP-B analogue present) were performed with PEEP (papers **II** and **III**), while the studies that were aimed to test surfactants containing phospholipids and two peptide analogues (paper **I**) or one analogue that is designed to combine the properties of both SP-C and SP-B (paper **IV**) were performed without PEEP. Even though the use of PEEP is extensive in the clinics to improve oxygenation and prevent alveolar collapse, the absence of PEEP can better assess the performance of a pulmonary surfactant. Previous studies have shown that a synthetic pulmonary surfactant containing all three essential elements (phospholipids, SP-B or SP-B variant and SP-C or SP-C variant) is able to achieve results similar to natural derived surfactants in the RDS rabbit model without PEEP (56, 57, 143), while the same surfactant lacking the SP-B or the SP-C element needs PEEP to achieve similar results (172, 183).

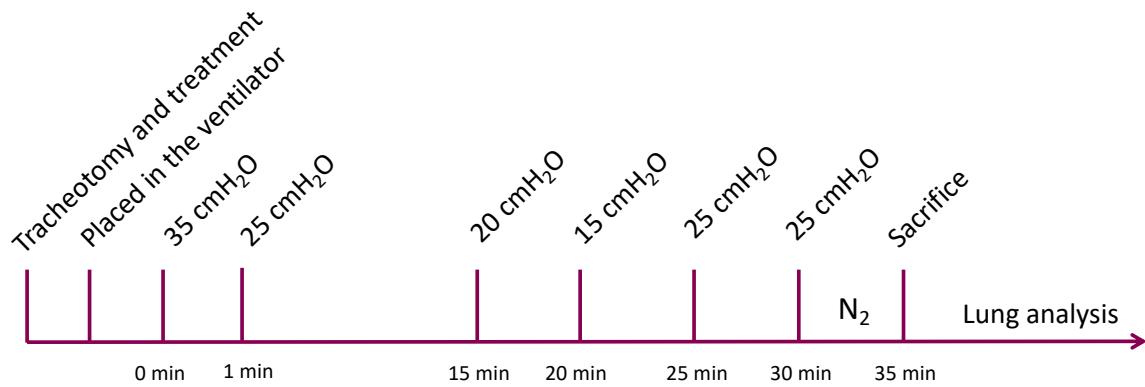


Figure 7. Timeline of the RDS animal experiments. The animals were tracheotomized and treatment was administered before placing the animals in the ventilator. The animals were ventilated during 30 min with air at different pressures followed by 5 min of ventilation with N₂ at 25 cmH₂O. The animals were sacrificed and the lungs were excised to measure the lung gas volumes.

ARDS rabbit model

An adult rabbit model of ARDS was developed in paper **III**. This animal model includes mild lung lavages that do not remove endogenous surfactant completely and induces an ARDS like state via ventilator induced lung injury (VILI), i.e. injurious ventilation that causes epithelial damage via high tidal volumes and low PEEP.

This model was used to test the ability of a synthetic surfactant based on only rSP-C33Leu to restore lung function and reduce inflammation after ARDS induction, and after treatment the animals were ventilated with physiological tidal volumes and PEEP.

Rabbit as model for RDS and ARDS

There is no perfect animal model that can be used to completely simulate a human lung disorder. However, there are some models that are better than others. Rabbits have been shown to be good candidates to simulate RDS and ARDS. The rabbit is phylogenetically closer to humans than mice or guinea pigs and cheaper than pigs and monkeys, and their relatively big size allows the study of lung mechanics even in premature fetuses. Anatomically the lungs of rabbits and humans are similar, with trachea, bronchi, bronchioles and alveoli but important differences are present. For example: the rabbit does not possess respiratory bronchioles, the

human lung is more vascularized than the rabbit lung, with seven capillaries per mm compared to five in rabbits, the human airway branching has less number of generations compared to the rabbit branching, and the two new airways generated in every branching have almost the same diameter in humans, but different diameters in rabbits (184).

3 RESULTS AND DISCUSSION

The development of synthetic surfactant preparations for the treatment of RDS has been ongoing for about 30-40 years. The research has evolved from the first synthetic surfactants that only contained phospholipids to mixtures of phospholipids with one or two surfactant protein analogues. In spite of all this effort, the development of synthetic surfactants is still a challenge. The native pulmonary surfactant is hard to replicate and produce because first, the lipid composition is very complex with multiple and diverse lipid species and second, the two hydrophobic lung surfactant proteins SP-B and SP-C are particularly hard to reproduce. During the last 30 years the main focus has been on the development and production of SP-B and SP-C analogues.

In paper I, we investigated the possibility to develop an SP-B analogue that lacks cysteines and therefore can not form disulfide bridges, for the reason that such analogues will likely be more straightforward to synthesize since inadvertent intermolecular disulfide pairings and need for Cys side-chain blocking groups are avoided. We evaluated *in vivo* the importance of the two disulfide bridges in an SP-B analogue called Mini-B27. Different versions of Mini-B27 with two, one or no intramolecular disulfide bridges (Figure 8) were mixed with the SP-C analogue SP-C33Leu and phospholipids, to form synthetic surfactants that were investigated in a premature rabbit fetus model of RDS. Curosurf® was used as positive control and untreated fetuses were used as an indication of immature animals that fulfill the criteria for RDS.

Mini-B27 was designed with the idea of creating an analogue similar to Mini-B, that was already proven to have good *in vivo* activity (56, 150), but containing a shorter and therefore probably more stable loop connecting the two α -helices. The stable turn could potentially maintain the hairpin structure of Mini-B27 even in the absence of the covalent disulfide bonds connecting the cysteine residues of each helix in Mini-B. The development of an active SP-B analogue without cysteines would be of interest because of the possibility to scale up its production in a cost-efficient manner.

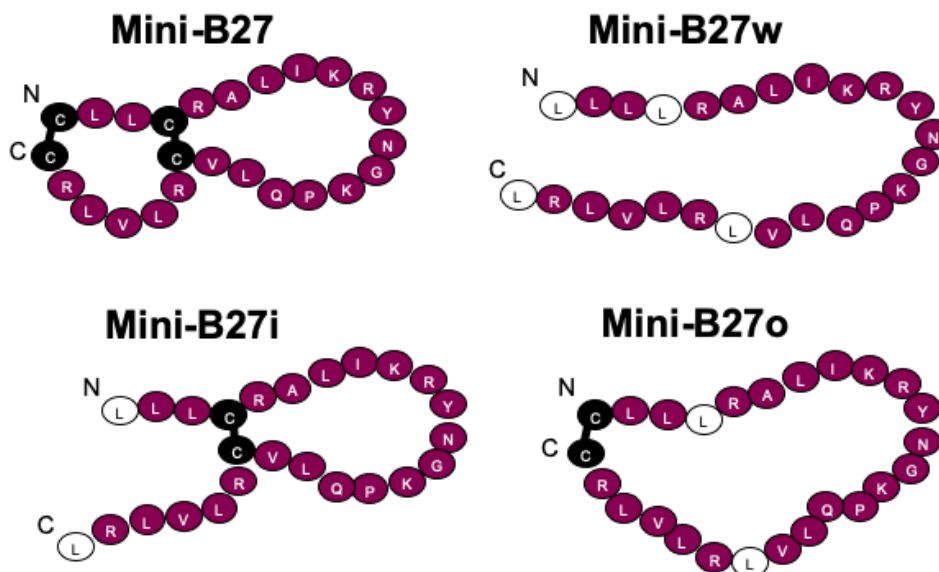


Figure 8: *Schematic representations of the four SP-B analogues and their different half-cystine content used in paper I. SP-B analogues containing two (Mini-B27), one inner or outer (Mini-B27i and Mini-B27o), or no (Mini-B27w) intramolecular disulfide bridge(s) were studied.*

Synthetic surfactant containing Mini-B27 with two intramolecular disulfide bridges was shown to give lung gas volumes similar to Curosurf® indicating a good ability to reduce alveolar surface tension and prevent alveolar collapse at the end of expiration. The synthetic surfactants containing Mini-B27 versions with one or no intramolecular disulfide bridges all showed an inferior ability to avoid alveolar collapse at the end of expiration (Figure 9). The results of paper I indicate that the presence of at least two disulfide bridges in Mini-B27 are important to create high lung gas volumes and avoid alveolar collapse at the end of expiration.

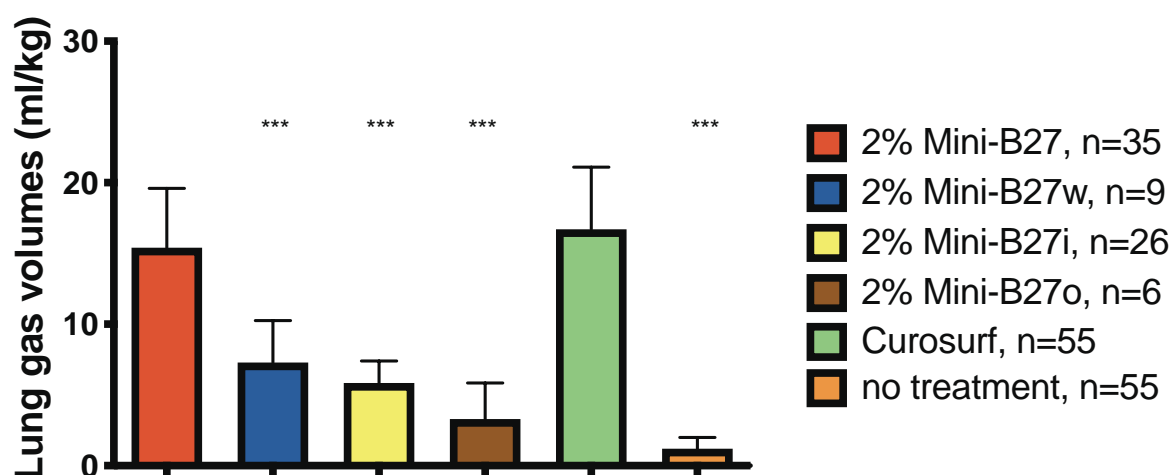


Figure 9. Lung gas volumes of the animals treated with pulmonary surfactants that included Mini-B27 analogues containing different disulfide patterns. Curosurf[®] was used as positive control and the untreated controls (no treatment) were used to confirm lack of sufficient endogenous surfactant in the animals. The synthetic surfactants contained phospholipids, 2% SP-C33Leu and 2% of Mini-B27, Mini-B27w, Mini-B27i, or Mini-B27o. The histograms and the error bars indicate the median values and the interquartile range, respectively. *** $p \leq 0.001$ against both Curosurf[®] and Mini-B27. Data from paper I. See Figure 8 for primary structures of the Mini-B27 analogues.

Another study, published a few months before paper I, developed an SP-B analogue based on Super Mini-B, called B-YL, where the cysteines were substituted by tyrosines suggesting that the hairpin structure could be maintained by aromatic interactions between the tyrosines (153). The *in vitro* and *in vivo* results shown by Walther et al. (153) differ from those in paper I in the sense that B-YL is as active as Curosurf[®], suggesting that the design of an active SP-B analogue lacking cysteines is indeed possible. There are many differences between the studies in paper I and Walther et al. (153), for example peptide design (Mini-B vs Super Mini-B), surfactant lipid composition and animal model, that could contribute to the different results obtained.

In paper II, we optimized the purification method of the recombinant SP-C analogue, rSP-C33Leu by including a chromatography step that removes lipid contaminants. The non-chromatographic purification of rSP-C33Leu previously described by Kronqvist et al. (172) efficiently isolated rSP-C33Leu from protein impurities, and by adding a two-step Lipidex-5000 chromatography we further removed non-protein contaminants that correspond to about 90% of the weight of the initially purified sample. The pure peptide was mixed with phospholipids (DPPC:egg yolk-PC:POPG in 50:40:10) to produce a surfactant that was then

tested in a premature rabbit fetus model of RDS (paper II) and in an adult rabbit model of ARDS (paper III).

In the rabbit model for neonatal RDS, the porcine derived surfactant Curosurf® was used as positive control, untreated fetuses were used to verify immaturity and presence of an RDS like state (very low lung compliance and low lung gas volumes at the end of the ventilation period). A mixture of only phospholipids (DPPC:egg yolk-PC:POPG in 50:40:10) was used to verify the importance of the presence of rSP-C33Leu. The premature rabbits treated with the surfactant containing 2% of rSP-C33Leu showed tidal volumes and lung gas volumes lower than the animals treated with Curosurf® but higher than the ones treated with only phospholipids (Figure 10). These results indicate that the synthetic surfactant containing rSP-C33Leu is able to improve the lung mechanics compared to a mixture of only phospholipids.

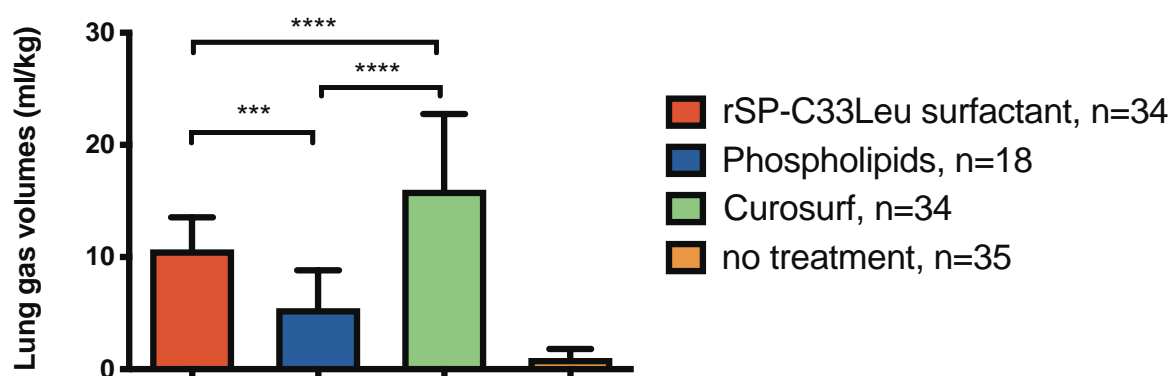


Figure 10: Lung gas volumes after treatment of a premature rabbit model of RDS with rSP-C33Leu surfactant and controls. Lung gas volumes calculated after ventilation with PEEP of untreated animals (no treatment), or animals treated with the synthetic surfactants containing phospholipids and rSP-C33Leu (rSP-C33Leu surfactant) or only phospholipids (Phospholipids), or the porcine derived surfactant Curosurf®. The histograms and the error bars indicate the median values and the interquartile range respectively. *** = $p \leq 0.001$ and **** = $p \leq 0.0001$. Data from paper II.

It is important to mention that the animal experiments described in paper II were performed using PEEP, which is required for optimal effect of surfactants that express only the properties of one surfactant protein and is commonly used in clinical treatment of RDS and ARDS patients. A synthetic surfactant created with phospholipids and 2% of the SP-C analogue SP-C33, which is structurally similar to SP-C33Leu (Table 5), was shown to give similar lung gas

volumes (median >10ml/kg) and tidal volumes as our rSP-C33Leu surfactant in the same RDS animal model when PEEP was applied (183). However, when PEEP was not applied the mixture of phospholipids and 2% SP-C33 showed significantly lower lung gas volumes (median <5 ml/kg) (56, 57). Also when SP-B or an analogue thereof was added to the mixture the lung gas volumes in the absence of PEEP were similar to the ones now obtained with our rSP-C33Leu surfactant in the presence of PEEP (median >10ml/kg) (56, 57, 143).

Even though an optimal effect of a synthetic surfactant is obtained only when compounds with properties of both SP-B and SP-C are present, rSP-C33Leu surfactant can be clinically relevant since it can achieve high lung gas volumes when PEEP is applied, and the possibility of producing SP-C33Leu recombinantly makes the surfactant production possible to scale up, for example to enable treatment of ARDS patients, as investigated in another rabbit model in paper **III**.

In paper **III**, synthetic pulmonary surfactant containing rSP-C33Leu was used in adult rabbits that were subjected to repeated bronchoalveolar lavages (BAL) followed by injurious ventilation in order to reduce oxygenation and induce an ARDS like state. Treatment with Curosurf[®] was used as a positive control and treatment with air as negative control. In this study we showed that the surfactant containing rSPC33Leu was able to increase oxygenation levels and reduce inflammation and edema in a similar manner as Curosurf[®]. Previous studies also investigated the use of synthetic surfactants in the treatment of ARDS showing an increase in oxygenation and dynamic compliance in lung-lavaged, surfactant deficient rats (141) and rabbits (140).

ARDS is characterized by capillary endothelium and alveolar epithelium damage, which leads to reduced alveolar surfactant activity and oxygenation, and creates an inflammatory state that leads to respiratory failure (185). In spite of that extensive research efforts have gone into evaluation of exogenous surfactant therapy to treat ARDS, clinical studies and meta-analysis thereof have failed to show improvement of mortality (186). However, we suggest that the activity of rSP-C33Leu surfactant observed in paper **III**, in combination with the possibility that its production could be scaled up, motivate further attempts to modify it so that resistance to inactivation by plasma components is increased. One possible way forward could be to screen for compounds that increase resistance to inactivation using *in vitro* surface activity measurements, and then study the most promising ones in the adult rabbit model of ARDS introduced in paper **III**. However, the results obtained in paper **IV** probably motivates to rather begin screening efforts for inhibitors of surfactant inactivation from an optimized Combo peptide-based surfactant. The results in paper **IV** showed that surfactants containing Combo

peptides are equal to natural derived surfactant also in the absence of PEEP, which is not the case for rSP-C33Leu surfactant.

In paper **IV**, we designed six different pulmonary surfactant protein analogues, called Combo peptides, based on the idea to combine properties of both SP-B and SP-C in one polypeptide chain. The Combo peptides were produced either by organic synthesis (Combo peptide 1) or by recombinant production (Combo peptides 2-6), and mixtures of each Combo peptide with phospholipids were tested in the rabbit model of neonatal RDS without PEEP. The architecture is similar for all Combo peptides; an N-terminal SP-B analogue part, followed by a linker and the SP-C analogue SP-C33Leu (Table 6). The sequence of the SP-B analogue and/or linker segment varied in the respective Combo peptides. The SP-B analogue in Combo peptide 1 was based on the first helix of Mini-BLeu, the sequence of Combo peptide 2 is based on Super Mini-B, and the sequences of Combo peptides 3-6 are based on full length Mini-BLeu. However, further modifications were introduced; in the Combo peptides all or half of the cysteines present in Mini-BLeu and Super Mini-B have been mutated to leucines to increase hydrophobicity, reduce complexity and avoid the formation of unwanted disulfide bridges. Combo peptides 1 and 3-5 do not contain any cysteine, while Combo peptides 3 and 6 contain 2 cysteines. Also, in Combo peptide 2 the methionines were mutated to leucines to avoid unintentional oxidation. The SP-B analogues present in Combo peptides 3 and 4 are similar to those in 6 and 5 respectively, however in Combo peptides 6 and 5 all the lysines have been substituted by arginines to avoid possible inadvertent acylation of the Lys side-chain amino group (187). Two linker versions were used, a short (GSG) in Combo peptides 1, 3-6 and a long (GSGSGSGS) in Combo peptide 2. The sequence of the SP-C analogue is constant in all Combo peptides and corresponds to SP-C33Leu that was studied in papers **II** and **III** with the exception of Combo peptides 5 and 6 where the lysines in SP-C33Leu were substituted by arginines (Table 6).

Table 6. Amino acid sequences of the Combo peptides studied in paper IV. The SP-B analogue parts are colored in light blue, the linkers in green and the SP-C analogue parts in dark blue. The amino acid residues are shown by their one letter code. The lysines mutated to arginines in Combo peptides 5 and 6 and the cysteines are marked in red.

	SP-B analogues	Linker	SP-C33Leu
Combo peptide 1	-----LWLLRALIKRIQALIPKG-----	GSG-----	IPSSPVHLKRLKLLLLLLLLLILLLILGALLLGL
Combo peptide 2	FPIPLPYLWLLRALIKRIQALIPKGGRLLPQLVLRVLRLS-	GSGSGSGS	IPSSPVHLKRLKLLLLLLLLLILLLILGALLLGL
Combo peptide 3	-----LWL ^C RALIKRIQALIPKGGRLLPQLV ^C RLVLRLS-	GSG-----	IPSSPVHLKRLKLLLLLLLLLILLLILGALLLGL
Combo peptide 4	-----LWLLRALIKRIQALIPKGGRLLPQLVLRVLRLS-	GSG-----	IPSSPVHLKRLKLLLLLLLLLILLLILGALLLGL
Combo peptide 5	-----LWLLRALI ^R RIQALIP ^R GGRLLPQLVLRVLRLS-	GSG-----	IPSSPVHL ^R RL ^R LLLLLLLLLILLLILGALLLGL
Combo peptide 6	-----LWL ^C RALI ^R RIQALIP ^R GGRLLPQLV ^C RLVLRLS-	GSG-----	IPSSPVHL ^R RL ^R LLLLLLLLLILLLILGALLLGL

The Combo peptides were designed with the idea of creating a synthetic pulmonary surfactant that contains only one protein component and a simple phospholipid mixture, and still is as efficient against RDS as animal derived surfactants like Curosurf[®]. The presence of only one protein component is beneficial from a regulatory point of view and could potentially reduce the cost of production of the pulmonary surfactant, allowing to expand its possible use to other lung diseases.

The Combo peptide 1 was made by chemical synthesis and Combo peptides 2-6 were produced and purified recombinantly. The purification procedure for Combo peptides 2-6 was based on the purification described for rSP-C33Leu in paper II, with some modifications. Hydrolysis and two-phase separation were introduced to hydrolyze phospholipids and isolate the target peptide from released fatty acids, glycerol, alcohols and phosphate groups. Also, the addition of Sephadex LH 60 chromatography enabled us to separate the peptide from unknown contaminant(s), which migrated as a lower band on SDS-PAGE and stained with Coomassie Brilliant Blue dye.

Each Combo peptide was mixed with phospholipids and the surfactants obtained were used in the premature rabbit model for RDS described above. Curosurf[®] was used as positive control and the mixture of phospholipid only was used as negative control. Also, animals that did not

receive any treatment were used as controls to confirm the lack of endogenous surfactant and RDS like state of each litter. The results showed that pulmonary surfactants containing 3% (w/w) of Combo peptide analogues achieve high lung gas volumes and high tidal volumes in the absence of PEEP (Figure 11). The absence of PEEP is a more demanding model and is thus an informative way of assessing the activity of the surfactant in our RDS rabbit model. It is also interesting to mention that in contrast to the results obtained in paper I, pulmonary surfactants containing Combo peptides 2 and 4 that lack cysteines are able to produce lung gas volumes and tidal volumes very similar to Curosurf®.

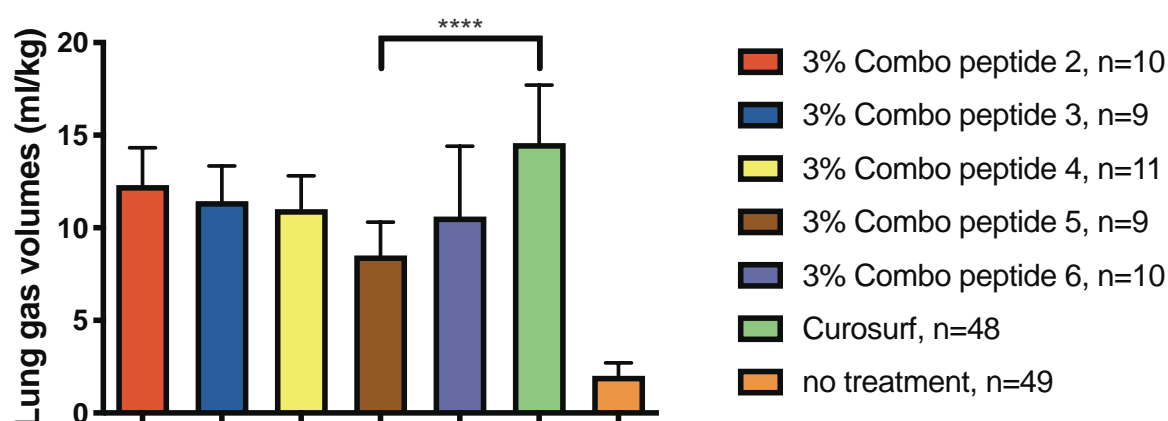


Figure 11. Lung gas volumes of animals after instillation of pulmonary surfactants containing 3% Combo peptide 2-6 and ventilation without PEEP. Curosurf was used as positive control and the untreated controls were used to detect lack of sufficient endogenous surfactant in the animals. The histograms and the error bars show the median and the interquartiles, respectively. **** = $p \leq 0.0001$. Data from paper IV.

Native mammalian pulmonary surfactant contains about 2% by dry weight of each hydrophobic surfactant protein (2% of SP-B and 2% of SP-C) which corresponds to about 1 molecule of dimerized SP-B to 4 molecules of SP-C. Natural derived surfactant preparations usually contain about 1% or less by dry weight of each surfactant protein (~0.5-1.1%), for example Curosurf® contains about 0.6% SP-B and 0.7% SP-C which corresponds to about 1 molecule of dimerized SP-B to 5 molecules of SP-C. The synthetic surfactant in clinical trials CHF5633 contains 0.2% of the SP-B analogue Mini-BLeu and 1.5% of the SP-C analogue SP-C33Leu, which in this case corresponds to about 1 molecule of Mini-BLeu per 7 molecules of SP-C33Leu. The synthetic production of SP-C33Leu and especially Mini-BLeu are

complicated and thus expensive, which probably affects the amount of protein analogues in CHF5633. The surfactants containing Combo peptides, per design, contain 1 SP-B analogue domain per 1 SP-C analogue domain, and a 3% Combo peptide surfactant that showed similar activity to Curosurf® contains about 1.5% by dry weight of each surfactant protein analogue domain. The protein quantity in the synthetic surfactants containing Combo peptides are thus comparable to native pulmonary surfactant.

There are not yet any structural data for the Combo peptides, but we hypothesize that the SP-B analogue domain and the SP-C analogue domain can interact independently with the phospholipids of surfactant. The Combo peptides could thus tentatively bind to different phospholipid (bi)layers (Figure 12), allowing membrane fusion, rapid transport between membranes and membrane stabilization during exhalation.

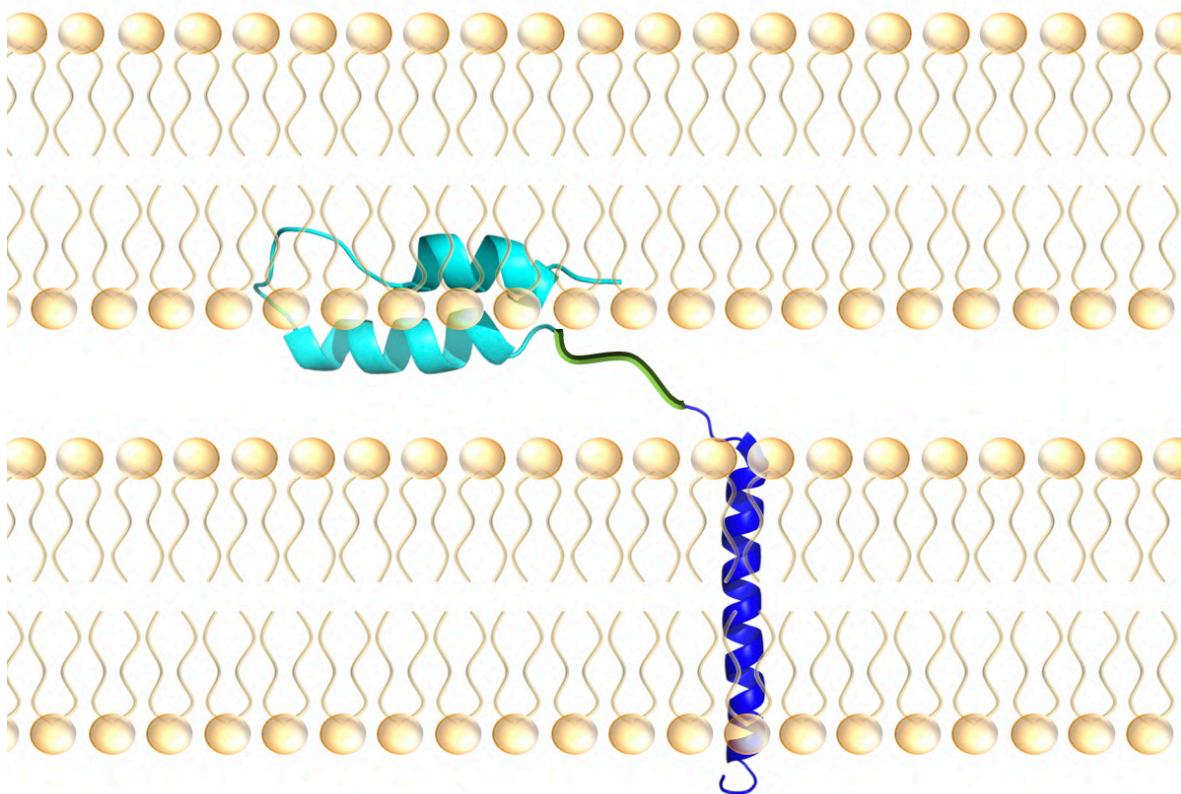


Figure 12. *Hypothetical model of a Combo peptide interacting with phospholipids of pulmonary surfactant. The SP-B analogue domain is represented by the Mini-B structure in light blue (pdb: 2DWF) the linker is shown as a green line and the SP-C analogue domain is illustrated by the SP-C33Leu structure in dark blue (pdb: 5NDA). Adapted from paper IV.*

Combo peptides and the possibility to produce them recombinantly is a potential breakthrough in the development of synthetic pulmonary surfactants. The production of recombinant SP-C analogues has already been reported, but the production of recombinant SP-B analogues has to our knowledge never been published, and in our hands recombinant expression of Mini-B using the NT* tag was unsuccessful (unpublished observations). The Combo peptides thus offer the first recombinant expression of a protein containing an SP-B analogue domain.

4 CONCLUSIONS

- A pulmonary surfactant containing 2% Mini-B27, 2% SP-C33Leu and phospholipids can achieve high lung gas volumes in a premature rabbit model of RDS, provided that the Mini-B27 peptide contains at least two intramolecular disulfide bridges.
- The purification of recombinant SP-C33Leu can be improved by adding a two step Lipidex-5000 chromatography that removes lipid contaminants.
- A pulmonary surfactant containing phospholipids and 2% of recombinant SP-C33Leu gives high lung gas volumes and tidal volumes in a premature rabbit model of RDS, as well as increased oxygenation and reduced inflammation and oedema in an adult rabbit model of ARDS.
- The production of recombinant Combo peptide analogues that harness properties of both SP-B and SP-C in one polypeptide chain is possible.
- Mixtures of 3% Combo peptide analogues with phospholipids give high tidal volumes and high lung gas volumes in a premature rabbit model of RDS in the absence of PEEP, which is comparable to the results obtained with a natural derived surfactant preparation and superior to those obtained by synthetic surfactant based on single surfactant peptide analogues.

5 FUTURE PRESPECTIVES

Despite the increasing knowledge on surfactant protein structure and function as well as the advances in development of hydrophobic surfactant protein analogues, there are still important questions that need to be resolved in order to enable rational design of synthetic surfactant. First, the specific activities and mechanisms of action of SP-B and SP-C are still not well understood, nor are their molecular interactions with surfactant phospholipids. Second, the 3D structure of native SP-B has not been resolved and there are severe limitations to the proposed structural models, mainly because SP-B differs in many ways from the proteins on which it has been modelled and only low-resolution structural data on SP-B is available. Third, the optimal phospholipid mixture that should be the base for synthetic surfactants is unknown. Fourth, an SP-B analogue that can be cost-efficiently produced and that lacks disulfide bonds to avoid intermolecular disulfides and complicated synthesis is not available. These are important research questions to tackle in the near future, not only to enable replacement of natural derived surfactant preparations for treatment of neonatal RDS, but also because the development of a cost-efficient synthetic surfactant could realize the since long held possibility to develop a surfactant for therapy of also other diseases such as ARDS, where high amounts of surfactant that is resistant to inactivation is needed, or lung infections where surfactant could be used as a carrier for drugs into the airways.

We believe that the work presented in this thesis brings us a bit closer to achieving some of these important goals. Even though there is still a lot of research to be done to optimize the preparations and understand the molecular behavior of the peptide analogues, the synthetic surfactants containing Combo peptides could potentially be used for both RDS and ARDS. The fact that these preparations contain only one peptide and that this peptide can be produced recombinantly could optimize the production yield and cost, resulting in a viable large-scale production. In addition, this synthetic surfactant could be modified by adding molecules that could increase the resistance to inactivation, such as SP-A and SP-D or, more likely, synthetic analogues thereof. Synthetic surfactant containing Combo peptides could also be used as a drug carrier by mixing it with for example antibiotics or antimicrobial peptides to treat lung infections. The drugs could thus be efficiently transported to the alveoli using the excellent spreading and adsorption properties of pulmonary surfactant, avoiding systemic effects and potentially reducing the amount of drug administered. Therefore, we speculate that surfactants containing Combo peptides could be used to treat RDS and other lung diseases.

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“When you are studying any matter, or considering any philosophy, ask yourself only what are the facts and what is the truth that the facts bear out. Never let yourself be diverted, either by what you wish to believe, or by what you think would have beneficent social effects if it were believed. [...] Love is wise, hatred is foolish. In this world, which is getting more and more closely interconnected, we have to learn to tolerate each other, we have to learn to put up with the fact that some people say things that we don’t like. We can only live together in that way—and if we are to live together and not die together—we must learn a kind of charity and a kind of tolerance, which is absolutely vital to the continuation of human life on this planet.”

Bertrand Russell (1872 - 1970)

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